CCLXIII. A COMPARATIVE STUDY OF THE PRODUCTION OF *l*-3:4-DIHYDROXYPHENYLALANINE FROM TYROSINE BY TYROSINASE FROM VARIOUS SOURCES

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IT has been shown previously [Raper, 1926] that when the monophenolase tyrosinase acts upon tyrosine the first product of the action is *l*-3:4-dihydroxyphenylalanine (dopa). Using qualitative methods and comparing different sources of the enzyme, it has since been found that the ease with which the production of dopa can be demonstrated varies considerably. With tyrosinase from mealworms, for instance, the production of dopa is easily shown, but not so with potato tyrosinase. It seemed desirable therefore to investigate the matter quantitatively, using different plant and animal tyrosinases, since the differences observed might be due to the presence of other enzymes. The present paper deals with this study, and it is shown that the variation in the dopa production from tyrosine is accounted for satisfactorily by the presence of contaminating enzymes which oxidize dopa but not tyrosine in the tyrosinase-tyrosine reaction. The ratio dopa produced/tyrosine oxidized has been used as a guide in the quantitative study of the production of dopa from tyrosine in the enzyme action. With the tyrosinases prepared from animal sources, mealworm and *Calliphora*, this ratio is consistently about five times greater than the same ratio using tyrosine from plant sources, potato or bran. This result is coincident with the fact that the plant enzyme preparations contain peroxidase, catechol oxidase and other polyphenol oxidases, whilst the animal enzymes do not contain peroxidase, but do contain catalase.

A certain amount of evidence has been brought forward that hydrogen peroxide or an organic peroxide is produced in the tyrosinase-tyrosine reaction. It would therefore be reasonable to expect them to be immediately activated by the peroxidase present. This peroxidase-peroxide system would oxidize the catechol derivative (dopa) produced by the action of the monophenolase on the monohydric phenol (tyrosine), resulting in the ratio dopa produced/tyrosine oxidized being much depressed.

The effect of the addition of a purified peroxidase preparation to the mealworm tyrosinase-tyrosine reaction strongly supports the above view. Peroxidase, when added to the animal tyrosinase-tyrosine system, in approximately equivalent concentration to the peroxidase content of the plant enzyme preparations, decreased the above ratio to the value obtained in the plant tyrosinase-tyrosine system. It is quite probable that the catechol oxidase described by Szent-Györgyi [1925] and Keilin [1928] also occurring in conjunction with the monophenolase will also affect the ratio. It is of great interest that peroxidase alone (without the addition of hydrogen peroxide), on addition to the animal tyrosinase-tyrosine system, decreases the accumulation of dopa considerably. Since peroxidase itself has no action on mono- or di-hydric phenols, this provides

additional evidence that either hydrogen peroxide, or an organic peroxide which is capable of being activated by peroxidase, is produced in the tyrosinase-tyrosine reaction.

Sources of enzymes used in the work, and method of preparation

Mealworm tyrosinase. This enzyme was prepared according to Raper's procedure [1926], and dialysed. It does not give any trace of a peroxidase reaction, but contains catalase.

Calliphora *tyrosinase.* 100 g. *Calliphora* larvae are ground with 200 ml. of 0·15% acetic acid. The hulls are strained, ground up with another 150 ml. acetic acid and again strained. The creamy fluid is centrifuged at once. The upper layers are rejected and the residue washed with 0·01% acetic acid. Finally the deposit is suspended in 350 ml. tap water together with 3 drops of ammonia (3%) and a little chloroform. Dialysed, it contains catalase as well as tyrosinase, but gives no reaction for peroxidase.

Potato tyrosinase. A dialysed press juice was used in many experiments, whilst in others the following purification process was used. To a volume of the potato juice, an equal volume of acetone-ether (3 : 1) was gradually added, with cooling and stirring. The flocculent, greyish precipitate was allowed to settle for 1 hr. in the ice-chest, the supernatant fluid was syphoned off and the precipitate collected, washed with acetone-ether and then with a little water. It was suspended in water, made faintly alkaline with ammonia, and toluene or chloroform added as antiseptic. The potato enzyme, whichever way it was made, contained peroxidase.

Bran tyrosinase. Prepared as described by Bertrand & Thomas [1920] with slight modifications. The bran is macerated with chloroform water and, after 5 hr. standing, strained through muslin. The liquid is centrifuged and the clear, supernatant liquid precipitated by the addition of acetone-ether (3 : 1). The precipitate is separated by centrifuging and washed again with alcohol or acetone-ether, suspended in water made faintly alkaline with ammonia and chloroform or toluene added. The preparation also contains peroxidase.

Peroxidase. Peroxidase was prepared from horse-radish as described by Bach & Chodat [1903].

Methods of estimation of tyrosine and dopa

(A) *Tyrosine.* (i) *Bromination method.* As described by Millar [1903] and modified by Raper & Wormall [1923].

(ii) *Colorimetric method.* As described by Folin & Ciocalteu [1927] based on Millon's reaction.

(B) *Dopa.* The methods which were found applicable are colorimetric and can be divided into two groups:

(*a*) those depending primarily on the formation of a red oxidation product;

(*b*) those depending on the reducing power of the catechol grouping.

(i) *Ammonium molybdate method.* Rae [1930] has used this reagent for adrenaline estimation. On examination of its applicability to dopa estimation, using ammonium molybdate (10%), the following facts were found. (*a*) The proportionality is good over the range 1/1000–1/25,000, but in lower dilutions the comparison is not easy to make on a Klett colorimeter, although the yellow colour is easily visible to the naked eye. (*b*) The standard and unknown must be buffered to the same *p*H before comparison is made, as the depth of colour varies slightly with the *p*H. All comparisons were therefore made in sodium acetate-acetic acid buffer at *p*H 5–7.

It should be noted that ascorbic acid gives a green coloration turning blue with the molybdate reagent, and hence the method is unreliable in its presence. All catechol derivatives can be estimated accurately by the method, provided that interfering substances are absent. Monohydric phenols do not give the reaction.

(ii) *The Vulpian reaction* [*1856*]. The development of a red colour when adrenaline is oxidized with iodine has been made a basis of colorimetric comparison by Schild [1933] and von Euler [1933]. The method can be applied satisfactorily to dopa as well. The procedure is as follows.

The reaction mixture is adjusted to *p*H 6·0 with phosphate buffer, and a preliminary determination to fix the strength of the control is made. *N*/100 Lugol solution (1 part I_2 to 2 parts KI) is added in proportion of about 1 ml. *N*/100 to 0·1 mg. dopa, until the colour is definitely brown. The solution is allowed to stand for a definite time (50–100 sec.), mixing well. At the end of this period an equal volume of thiosulphate (*N*/100) is added to destroy the excess iodine and the solution made up to a convenient volume with buffer and compared in the colorimeter. The method gives good results down to a concentration of 1/100,000; below this it is hard to make the comparison with an ordinary Klett colorimeter. The test is not affected by ascorbic acid (so long as excess of iodine is added) and it is not given by tyrosine.

The separate estimation of tyrosine and dopa in a mixture. Tyrosine is not very soluble in water near neutrality (1 in 2000), and the yield of dopa in the enzyme action is exceedingly small when starting with 200 ml. saturated tyrosine solution (0·1 g.) since only about 50% of the tyrosine is oxidized. A method of concentration is therefore advisable.

The method used by Raper [1926] for the isolation of *l*-dopa from the tyrosinase-tyrosine reaction was tested on a quantitative basis.

Addition of excess lead acetate to a neutral solution of tyrosine and dopa gives no precipitate. If, by cautious addition of dilute ammonia (1%), the solution is made distinctly but not strongly alkaline to litmus, *p*H 8·5, a yellow precipitate of the lead compound of dopa is obtained, the tyrosine remaining in solution. An excess of ammonia must be avoided, as, if *p*H 9 is exceeded there is a danger of tyrosine being slowly precipitated. By allowing the precipitate to settle (about 3 hr. are necessary) and filtering gravimetrically, a complete separation of dopa and tyrosine is obtained. The lead-dopa compound is easily decomposed by suspension in water made faintly acid with acetic acid and saturating with H_2S. Excess lead is removed from the mother-liquor (containing the tyrosine) by H_2S. The lead-dopa precipitate is best filtered through tightly packed fine asbestos, and the whole taken up again and washed into a conical flask with ground glass stopper. After decomposition of the lead precipitate and thorough washing by suspension in water, the H_2S must be completely removed by boiling or aeration *in vacuo*. A drop of sulphurous acid prevents any oxidation of the dopa if the solution is boiled. The solution, after decomposition of the lead-dopa precipitate and neutralization with acetate buffer, is brought to a convenient volume, e.g. 25 or 50 ml., and the comparison made with the standard.

The mother-liquor containing the tyrosine is also treated with H_2S, filtered, washed and, after removal of H_2S by boiling, made up to convenient bulk. The tyrosine is estimated by bromination.

In a trial of the method, using 20 ml. of a solution containing 20 mg. dopa and 5 mg. tyrosine, the amounts found were: dopa, 19 mg.; tyrosine, 5·1 mg. To obtain further experience of the method before applying it in the enzyme

action, the production of dopa by the oxidation of tyrosine with Fenton's reagent [Raper, 1932] was studied.

A comparison of the yields in neutral and acid solution was made, using varying amounts of hydrogen peroxide. The reactants were dissolved in 200 ml. water and kept overnight at room temperature. The dichromate test for H_2O_2 was then negative. The results are given in Table I.

Table I

Acetic acid 10% ml.	$FeSO_4$ g.	Tyrosine mg.	H_2O_2 6% mol.	Yield of dopa mg.
0·5	0·05	100	1·0	20·4
—	0·05	100	1·0	19·8
0·5	0·05	100	1·5	25·3
—	0·05	100	1·5	24·0
0·5	0·05	100	2·0	22·5

The highest yield of dopa is about 25% of the tyrosine taken. The tyrosine left, if any, was not estimated in these experiments, but the filtrate gave a weak Millon's test—showing that some was still present. The reaction appeared to go equally well in acid and neutral solution.

Quantitative investigations on the tyrosinase-tyrosine reaction

The enzyme actions were carried out in all cases, unless otherwise stated, in a thermostat at 25°. The general procedure was as follows. The flasks (identical 500 ml.) were immersed bodily in the thermostat; oxygen was used for aeration, the gases being first drawn through a toluene-water mixture immersed in the thermostat. All solutions were brought to the temperature of the thermostat before mixing and the reaction flasks were gently shaken mechanically. Toluene, together with a few drops of capryl alcohol, were added.

The *p*H of the reaction mixtures was quickly found by means of the hydrogen electrode, or using Walpole's comparative method with a series of buffers. All the enzyme reactions were timed, usually lasting 2–5 hr.

To stop the reaction, acetic acid (5 ml. 10%) and lead acetate (5 ml. 20%) were added—the enzyme is thus precipitated and the *p*H is below 5. The indole derivatives were allowed to oxidize to melanin by plugging the flasks with cotton-wool and keeping overnight. The melanin could then easily be filtered off, leaving a perfectly clear solution containing the dopa and unoxidized tyrosine.

The procedure for separating the dopa and tyrosine described above was used, dopa being estimated colorimetrically and tyrosine by the bromination method. Controls were always done at the same time with distilled water (same volume as that of substrate) and the same amount of enzyme. This is important, to get the blank tyrosine figure when using the bromination method, especially with the potato enzyme. The blank dopa estimation was always found to be zero.

(i) *Effect of initial* p*H on the dopa produced.* Raper & Wormall [1923] found that the limits of tyrosinase activity were *p*H 5–10, and that more tyrosine was oxidized by the enzyme at *p*H 8 than at *p*H 7 and less still at *p*H 6, using the potato enzyme. No clear optimum was observed, although the production of "red quinone" was best at *p*H 6·5. Narayanamurti & Ayyar [1929] found that the tyrosinase from *Dolichos lab lab* had an optimum at *p*H 6·5, and Nobutani [1936] with potato enzyme found an optimum at *p*H 7·38. Graubard & Nelson [1935] found that with potato tyrosinase, when purified by different methods, there was a narrowing of *p*H range of activity on purification, the optimum

being *p*H 6·5–7. The results indicate that the figures obtained by various investigators depend on the isoelectric point of the proteins with which the enzyme is associated.

It was important for the present investigation to know what effect, if any, the *p*H had on the ratio dopa produced/tyrosine oxidized. The reaction mixtures were adjusted before starting to *p*H 6, 7 and 8 by addition of dilute acetic acid and/or ammonia.

Experimental solutions. Three identical 500 ml. flasks each containing tyrosine (200 ml., i.e. 100 mg.) and mealworm enzyme (10 ml.) adjusted to *p*H 6, 7 and 8.

Control solution. 200 ml. distilled water, 10 ml. mealworm enzyme. Duration of aeration (oxygen), 4·5 hr. From preliminary experiments it was found that adjustment of the *p*H of the controls 6–8 did not make any difference to the tyrosine figure and only one control was therefore necessary.

Table II

*p*H of solution at start	Tyrosine oxidized mg.	Dopa produced mg.	Dopa produced / Tyrosine oxidized
6	34·1	3·518	0·105
7	45·9	4·219	0·093
8	54·8	5·517	0·101

The results of Table II show that the ratio is independent of *p*H within the limits *p*H 6–8. Incidentally, it confirms Raper & Wormall's results [1923] when working with potato enzyme. They found that most tyrosine disappeared at *p*H 8, and least at *p*H 6.

(ii) *Quantitative comparison of enzymes from different sources with respect to the dopa produced.* For a comparison of tyrosinases from different sources as regards dopa production it is desirable to start with enzyme preparations of about equal activities on the substrate.

Graubard [1932] found that the most convenient method for comparison of enzyme strengths was to follow the oxygen uptakes, using a variety of suitable substrates. Upon this basis Richter [1934] and Graubard & Nelson [1935] have established "tyrosinase units".

In this work Barcroft respirometers were used to estimate the activities of the enzyme preparations. As substrates, were used: (*a*) tyramine hydrochloride (0·4 %) + dopa (0·008 %)—to prevent the initial lag; (*b*) *p*-cresol (0·25 %).

The respirometer bottles contained:

	Right-hand ml.	Left-hand ml.
KOH (40 %) in cup	0·1	0·1
Enzyme	0·5	0·5
Substrate	0·5	—
Phosphate buffer, *p*H 7	2·0	2·5
Total vol.	3·1	3·1

The taps of the respirometer were closed after 5 min. equilibration in the thermostat. Oscillations were about 90 per min. The slope of the oxygen uptake curve with time was used as an index of the activity of the enzyme preparations and, by means of this, approximately equivalent amounts of the enzyme could be taken for the dopa-production experiments with tyrosine.

Conditions of experiment. Substrate employed, 250 ml. 1/2000 tyrosine; enzyme 12·5 ml.; *p*H 7; time of aeration (oxygen) 4 hr. Controls were done with all enzymes and the tyrosine and dopa then assayed.

Table III summarizes the results obtained using four different sources of tyrosinase. The difference in the ratio dopa produced/tyrosine oxidized is pronounced.

Table III

Enzyme	Tyrosine oxidized mg.	Dopa produced mg.	Dopa produced / Tyrosine oxidized
Exp. 1. Mealworm enzyme	73·3	8·3	0·113
Potato enzyme	56·6	2·1	0·037
Exp. 2. Mealworm enzyme*	66·9	10·0	0·15
Potato enzyme	79·6	2·1	0·026
Exp. 3. *Calliphora* enzyme	58·5	7·02	0·12
Bran enzyme	49·7	1·25	0·025

* Same enzyme solutions as Exp. 1, 2 weeks later.

(iii) *Effect of peroxidase on the mealworm tyrosinase-tyrosine reaction. Standardization of peroxidase from horse-radish and the "peroxidase" content of potato enzyme.* The strength of the peroxidase in the potato enzyme and of the horse-radish preparation was estimated (*a*) by Willstätter's purpurogallin method and (*b*) by using guaiacol as substrate.

Method (*a*). 0·2 ml. of the peroxidase preparation yielded 0·185 g. purpurogallin. 2·0 ml. of filtered potato enzyme yielded 0·155 g. purpurogallin. Thus 1·0 ml. peroxidase was equivalent to 12 ml. of the potato enzyme.

Method (*b*). *Guaiacol standardization.* Since tyrosinase also acts on pyrogallol giving purpurogallin [Pugh & Raper, 1927], the above method would appear to give the sum of the peroxidase and tyrosinase contents. However, as the concentration of hydrogen peroxide is appreciable, and Pugh [1930] has shown that tyrosinase is inhibited by high concentrations of H_2O_2, it may well be that tyrosinase action is inhibited altogether in this experiment. It was thought advisable to check this point using as substrate guaiacol upon which tyrosinase has no action. Both enzymes were diluted ten times. As substrate, 5 ml. of a 1 % solution of guaiacol in water and 1 ml. of 4 % hydrogen peroxide were used.

(*a*) Peroxidase preparation ml.	(*b*) Potato enzyme ml.	Remarks
0·05	0·5	After 5 min. red colour in (*a*) slightly darker
0·05	0·8	Colour approx. the same on development in (*a*) and (*b*)

1 ml. peroxidase preparation is thus equivalent to between 10 and 16 ml. potato enzyme. Hence this method gives approximately the same result as the Willstätter method.

Effect of peroxidase on the dopa produced in the mealworm tyrosinase-tyrosine reaction. The peroxidase was added in equivalent concentration to the amount found in the potato enzyme.

Exp. 1. Solutions: (*a*) Tyrosine (250 ml. 1/2000); mealworm enzyme (12·5 ml.); adjusted to *p*H 7. (*b*) Tyrosine (250 ml.); mealworm enzyme (12·5 ml.); peroxidase preparation (2·0 ml.); adjusted to *p*H 7. (*c*) and (*d*) Controls of (*a*) and (*b*) using H_2O (250 ml.) instead of substrate. (*e*) Tyrosine (250 ml.); peroxidase (2·0 ml.); adjusted to *p*H 7. (*f*) Tyrosine (250 ml.); mealworm enzyme (12·5 ml.); boiled peroxidase (2 ml.). Aeration time (oxygen) 5 hr.; toluene and capryl alcohol added as usual.

Exp. 2. Exactly similar to Exp. 1, but instead of using 2 ml. peroxidase preparation, the quantity was reduced to 0·5 ml. All controls were done in an identical manner.

After completion of the enzyme action, acetic acid and lead acetate were added as usual and the dopa and tyrosine assayed.

Table IV

Exp.	Enzyme	Tyrosine oxidized mg.	Dopa produced mg.	Dopa produced / Tyrosine oxidized
1	Mealworm enzyme alone	33·4	6·5	0·194
	Mealworm enzyme and peroxidase (2 ml.)	36·1	1·9	0·053
	Mealworm enzyme and boiled peroxidase (2 ml.)	29·1	6·25	0·21
2	Mealworm enzyme alone	30·9	5·4	0·14
	Mealworm enzyme and peroxidase (0·5 ml.)	33·4	1·6	0·05
	Mealworm enzyme and boiled peroxidase (0·5 ml.)	32·76	6·0	0·18

From the results recorded, the conclusion seems warranted that the behaviour of tyrosinase preparations (monophenolases) from different sources, as regards dopa accumulation, depends on the presence of enzymes other than the monophenolase, which oxidize the dopa. They are possibly peroxidases or catechol oxidases, since it has been shown that peroxidase itself will bring about a lowering of the ratio dopa produced/tyrosine oxidized down to the level observed consistently with the potato enzyme.

Summary

1. A method for estimating *l*-3:4-dihydroxyphenylalanine (dopa) and tyrosine in the presence of each other is described.

2. The amount of dopa produced when tyrosine is oxidized by tyrosinase varies with the source of the enzyme.

3. Evidence is presented that diminution in the production of dopa is due to the presence of peroxidase or polyphenolases in the tyrosinase preparations.

4. The effect of peroxidase in diminishing dopa production indicates that hydrogen peroxide or an organic peroxide capable of acting with peroxidase is produced in the oxidation of tyrosine by tyrosinase.

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